

TITLE OF THE INVENTION

NUCLEOTIDE SEQUENCES WHICH CODE FOR THE rpsL GENE

BACKGROUND OF THE INVENTION

5 Field of the Invention

The present invention provides nucleotide sequences from coryneform bacteria which code for the rpsL gene and a process for the fermentative preparation of amino acids using bacteria in which the rpsL gene is enhanced.

Background of the Invention

10 L-Amino acids, in particular L-lysine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry, and, very particularly, in animal nutrition.

15 It is known that amino acids are prepared by fermentation from strains of coryneform bacteria, in particular *Corynebacterium glutamicum*. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as, for example, stirring and supply of oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the 20 microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites or are auxotrophic for metabolites of regulatory importance and produce amino acids are obtained in this manner.

25 Methods of the recombinant DNA technique have also been employed for some years for improving the strain of *Corynebacterium* strains which produce L-amino acid, by amplifying individual amino acid biosynthesis genes and investigating the effect on the amino acid production. However, there is a continuing need for new methods of producing L-amino acids.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide new methods for an improved fermentative preparation of amino acids.

The present invention is based on the discovery that bacteria in which the rpsL gene is enhanced can be used to fermentatively produce amino acids.

Accordingly, the invention provides an isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the rpsL gene chosen from the group consisting of a

- a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
- b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,
- c) polynucleotide which is complementary to the polynucleotides of a) or b), and
- d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of the ribosomal protein S12.

The present invention also provides the above-mentioned polynucleotide, this preferably being a DNA which is capable of replication, comprising:

- (i) the nucleotide sequence shown in SEQ ID no. 1, or
- (ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or
- (iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and optionally
- (iv) sense mutations of neutral function in (i) which do not modify the activity of the protein/polypeptide

In addition, the present invention also provides polynucleotides chosen from the group consisting of

- a) polynucleotides comprising at least 15 successive nucleotides chosen from the nucleotide sequence of SEQ ID No. 1 between positions 1 and 499,
- b) polynucleotides comprising at least 15 successive nucleotides chosen from the

nucleotide sequence of SEQ ID No. 1 between positions 500 and 883,

c) polynucleotides comprising at least 15 successive nucleotides chosen from the nucleotide sequence of SEQ ID No. 1 between positions 884 and 1775.

The present invention also provides

a polynucleotide, in particular DNA, which is capable of replication and comprises the nucleotide sequence as shown in SEQ ID No. 1;

a polynucleotide which codes for a polypeptide which comprises the amino acid sequence as shown in SEQ ID No. 2;

a vector containing the polynucleotide according to the invention, in particular a shuttle vector or plasmid vector, and

coryneform bacteria which contain the vector or in which the *rpsL* gene is enhanced.

In addition, the present invention also provides the *Corynebacterium glutamicum* strain DM1545 deposited as DSM 13992 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany).

The present invention also provides a process for the fermentative preparation of an L-amino acid, comprising:

- a) fermenting coryneform bacteria which produce the L-amino acid and in which at least the *rpsL* gene or nucleotide sequences which code for it are enhanced,
- b) concentrating the L-amino acid in the medium or in the cells of the bacteria, and
- c) isolating the L-amino acid.

The present invention also provides a process for discovering RNA, cDNA and DNA in order to isolate nucleic acids or polynucleotides or genes which code for the ribosomal protein S12 or have a high similarity with the sequence of the rpsL gene, which comprises employing the polynucleotide comprising the polynucleotide sequences as claimed in claim 1 as a hybridization probe.

The present invention additionally provides a process for identifying a nucleic acid which codes for the ribosomal protein S12 or have a high similarity with the sequence of the *rpsL* gene, comprising:

contacting a sample with the polynucleotide sequence as claimed in claim 1 under conditions under hybridization conditions such that the polynucleotide sequence as claimed

in claim 1 hybridizes with said nucleic acid when said nucleic acid is present in the sample.

Further, the present invention additionally provides:

a DNA which originates from coryneform bacteria and codes for ribosomal S12 proteins, wherein the associated amino acid sequences between positions 38 to 48 in SEQ ID No. 2 are modified by amino acid exchange;

5 a DNA which originates from coryneform bacteria and codes for ribosomal S12 proteins, wherein the associated amino acid sequences at position 43 in SEQ ID No. 2 contain any other proteinogenic amino acid excluding L-lysine; and

10 a DNA which originates from coryneform bacteria and codes for ribosomal S12 proteins, wherein the associated amino acid sequences at position 43 in SEQ ID No. 2 contain L-histidine or L-arginine.

15 The invention also provides polynucleotides which substantially comprise a polynucleotide sequence, which are obtainable by screening by means of hybridization of a corresponding gene library of a coryneform bacterium, which comprises the complete gene or parts thereof, with a probe which comprises the sequence of the polynucleotide according to the invention according to SEQ ID No.1 or a fragment thereof, and isolation of the polynucleotide sequence mentioned.

20 Polynucleotides which comprise the sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate, in the full length, nucleic acids or polynucleotides or genes which code for the ribosomal protein S12 or to isolate those nucleic acids or polynucleotides or genes which have a high similarity with the sequence of the rpsL gene. They can also be applied as a probe on so-called "arrays", micro arrays" or "DNA chips" in order to detect and determine the corresponding polynucleotides or sequences derived therefrom, such as e.g. RNA or cDNA.

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BRIEF DESCRIPTION OF THE FIGURES

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same becomes better understood by reference to the following Figure in conjunction with the detailed description below.

Figure 1: plasmid pK18mobsacB_rpsL-1545.

DETAILED DESCRIPTION OF THE INVENTION

Where L-amino acids or amino acids are mentioned herein, this means one or more amino acid, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Lysine is particularly preferred.

When L-lysine or lysine are mentioned herein, not only the bases but also the salts, such as e.g. lysine monohydrochloride or lysine sulfate, are encompassed term by this.

Polynucleotides which comprise the sequences according to the invention are furthermore suitable as primers with the aid of which DNA of genes which code for the ribosomal protein S12 can be prepared by the polymerase chain reaction (PCR). Such oligonucleotides which serve as probes or primers comprise at least 25, 26, 27, 28, 29 or 30, preferably at least 20, 21, 22, 23 or 24, very particularly preferably at least 15, 16, 17, 18 or 19 successive nucleotides. Oligonucleotides with a length of at least 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40, or at least 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides are also suitable. Oligonucleotides with a length of at least 100, 150, 200, 250 or 300 nucleotides are optionally also suitable.

"Isolated" means separated out of its natural environment. "Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

The polynucleotides according to the invention include a polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom and also those which are at least in particular 70% to 80%, preferably at least 81% to 85%, particularly preferably at least 86% to 90%, and very particularly preferably at least 91%, 93%, 95%, 97% or 99% identical to the polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom.

"Polypeptides" are understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, in particular those with the biological activity of the ribosomal protein S12 and also those which are at least 70% to 80%, preferably at least 81% to 85%, particularly preferably at least 86% to 90%, and very particularly preferably at least 91%, 93%, 95%, 97% or 99% identical to the polypeptide according to SEQ ID No. 2 and have the activity described

above.

The invention furthermore relates to a process for the fermentative preparation of amino acids chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, 5 L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine using coryneform bacteria which in particular already produce amino acids and in which the nucleotide sequences, preferably endogenous, which code for the rpsL gene are enhanced, in particular over-expressed.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or using a gene or allele which codes for a corresponding enzyme or protein with a high activity, and optionally combining these measures. By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 10 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

The microorganisms provided by present invention can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and 15 ethanol. They can be representatives of coryneform bacteria, in particular of the genus *Corynebacterium*. Of the genus *Corynebacterium*, there may be mentioned in particular the species *Corynebacterium glutamicum*, which is known among those skilled in the art for its 20 ability to produce L-amino acids.

Suitable strains of the genus *Corynebacterium*, in particular of the species 25 *Corynebacterium glutamicum* (*C. glutamicum*), are in particular the known wild-type strains
Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium thermoaminogenes FERM BP-1539
30 Corynebacterium melassecola ATCC17965
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 and

Brevibacterium divaricatum ATCC14020

and L-amino acid-producing mutants or strains prepared therefrom, such as, for example, the L-lysine-producing strains

Corynebacterium glutamicum FERM-P 1709
5 Brevibacterium flavum FERM-P 1708
Brevibacterium lactofermentum FERM-P 1712
Corynebacterium glutamicum FERM-P 6463
Corynebacterium glutamicum FERM-P 6464
Corynebacterium glutamicum DM58-1
10 Corynebacterium glutamicum DG52-5
Corynebacterium glutamicum DSM5715 and
Corynebacterium glutamicum DSM12866.

The new rpsL gene from *C. glutamicum* which codes for the ribosomal protein S12 has been isolated as described herein.

To isolate the rpsL gene or also other genes of *C. glutamicum*, a gene library of this microorganism is first established in *Escherichia coli* (*E. coli*). The establishment of gene libraries is described in generally known textbooks and handbooks. The textbook by Winnacker: Gene und Klone, Eine Einführung in die Gentechnologie [Genes and Clones, An Introduction to Genetic Engineering] (Verlag Chemie, Weinheim, Germany, 1990), or the handbook by Sambrook et al.: Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989) may be mentioned as an example. A well-known gene library is that of the *E. coli* K-12 strain W3110 set up in λ vectors by Kohara et al. (Cell 50, 495-508 (1987)). Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) describe a gene library of *C. glutamicum* ATCC13032, which was established with the aid of the 20 cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) in the *E. coli* K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575).

Börmann et al. (Molecular Microbiology 6(3), 317-326) (1992)) in turn describe a 25 gene library of *C. glutamicum* ATCC13032 using the cosmid pHG79 (Hohn and Collins,

Gene 11, 291-298 (1980)).

To prepare a gene library of *C. glutamicum* in *E. coli* it is also possible to use plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979)) or pUC9 (Vieira et al., 1982, Gene, 19:259-268). Suitable hosts are, in particular, those *E. coli* strains which are restriction- and recombination-defective. An example of these is the strain DH5 α mcr, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649). The long DNA fragments cloned with the aid of cosmids can in turn be subcloned in the usual vectors suitable for sequencing and then sequenced, as is described e.g. by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977).

The resulting DNA sequences can then be investigated with known algorithms or sequence analysis programs, such as e.g. that of Staden (Nucleic Acids Research 14, 217-232(1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

The new DNA sequence of *C. glutamicum* which codes for the rpsL gene and which, as SEQ ID No. 1, is a constituent of the present invention has been found. The amino acid sequence of the corresponding protein has furthermore been derived from the present DNA sequence by the methods described above. The resulting amino acid sequence of the rpsL gene product is shown in SEQ ID No. 2. It is known that enzymes endogenous in the host can split off the N-terminal amino acid methionine or formylmethionine of the protein formed.

Coding DNA sequences which result from SEQ ID No. 1 by the degeneracy of the genetic code are also a constituent of the invention. In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Conservative amino acid exchanges, such as e.g. exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are furthermore known among experts as "sense mutations" which do not lead to a fundamental change in the activity of the protein, i.e. are of neutral function. Such mutations are also called, *inter alia*, neutral substitutions. It is furthermore known that changes on the N and/or C terminus of a protein cannot substantially impair or can even stabilize the function thereof. Information in this context can be found by the expert, *inter alia*, in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) and in known textbooks of

genetics and molecular biology. Amino acid sequences which result in a corresponding manner from SEQ ID No. 2 are also a constituent of the invention.

In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers which result from SEQ ID No. 1 are a constituent of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

Instructions for identifying DNA sequences by means of hybridization can be found by the expert, *inter alia*, in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology (1991) 41: 255-260). The hybridization takes place under stringent conditions, that is to say only hybrids in which the probe and target sequence, i.e. the polynucleotides treated with the probe, are at least 70% identical are formed. It is known that the stringency of the hybridization, including the washing steps, is influenced or determined by varying the buffer composition, the temperature and the salt concentration. The hybridization reaction is preferably carried out under a relatively low stringency compared with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

A 5x SSC buffer at a temperature of approximately 50°C - 68°C, for example, can be employed for the hybridization reaction. Probes can also hybridize here with polynucleotides which are less than 70% identical to the sequence of the probe. Such hybrids are less stable and are removed by washing under stringent conditions. This can be achieved, for example, by lowering the salt concentration to 2x SSC and optionally subsequently 0.5x SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995) a temperature of approximately 50°C - 68°C being established. It is optionally possible to lower the salt concentration to 0.1x SSC. Polynucleotide fragments which are, for example, at least 70% or at least 80% or at least 90% to 95% identical to the sequence of the probe employed can be isolated by increasing the hybridization temperature stepwise from 50°C to 68°C in steps of approximately 1 - 2°C. Further instructions on hybridization are obtainable on the market in the form of so-called kits (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany, Catalogue No. 1603558).

Instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) can be found by the expert, inter alia, in the handbook by Gait:

Oligonucleotide synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

- 5 It has been found that coryneform bacteria produce amino acids in an improved manner after enhancement of the rpsL gene.

To achieve an over-expression, the number of copies of the corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible to increase the expression in the course of fermentative amino acid production. The expression is likewise improved by measures to prolong the life of the m-RNA. Furthermore, the enzyme activity is also increased by preventing the degradation of the enzyme protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, 10 or can be integrated and amplified in the chromosome. Alternatively, an over-expression of 15 the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Instructions in this context can be found by the expert, inter alia, in Martin et al. (Bio/Technology 5, 137-146 (1987)), in Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), in Eikmanns et al. (Gene 102, 93-98 20 (1991)), in European Patent Specification 0 472 869, in US Patent 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)), in Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)), in LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), in Patent Application WO 96/15246, in Malumbres et al. (Gene 134, 25 15 - 24 (1993)), in Japanese Laid-Open Specification JP-A-10-229891, in Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)), in Makrides (Microbiological Reviews 60:512-538 (1996)) and in known textbooks of genetics and molecular biology.

By way of example, for enhancement the rpsL gene according to the invention was 30 over-expressed with the aid of episomal plasmids. Suitable plasmids are those which are replicated in coryneform bacteria. Numerous known plasmid vectors, such as e.g. pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554), pEKEx1

(Eikmanns et al., Gene 102:93-98 (1991)) or pHS2-1 (Sonnen et al., Gene 107:69-74 (1991)) are based on the cryptic plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors, such as e.g. those based on pCG4 (US-A 4,489,160), or pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990)), or pAG1 (US-A 5,158,891), can be used in the same manner.

Plasmid vectors which are furthermore suitable are also those with the aid of which the process of gene amplification by integration into the chromosome can be used, as has been described, for example, by Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)) for duplication or amplification of the hom-thrB operon. In this method, the complete gene is cloned in a plasmid vector which can replicate in a host (typically E. coli), but not in *C. glutamicum*. Possible vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pGEM-T (Promega Corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-84; US-A 5,487,993), pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)), pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516) or pBGS8 (Spratt et al., 1986, Gene 41: 337-342). The plasmid vector which contains the gene to be amplified is then transferred into the desired strain of *C. glutamicum* by conjugation or transformation. The method of conjugation is described, for example, by Schäfer et al.

(Applied and Environmental Microbiology 60, 756-759 (1994)). Methods for transformation are described, for example, by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a "cross over" event, the resulting strain contains at least two copies of the gene in question.

It has furthermore been found that amino acid exchanges in the section between position 38 to 48 of the amino acid sequence of the ribosomal protein S12 shown in SEQ ID No. 2 improve the lysine production of coryneform bacteria.

Preferably, L-lysine at position 43 is exchanged for any other proteinogenic amino acid excluding L-lysine, exchange for L-histidine or L-arginine being preferred. Exchange for L-arginine is very particularly preferred.

The base sequence of the allele rpsL-1545 contained in strain DM1545 is shown in SEQ ID No. 3. The rpsL-1545 allele codes for a protein, the amino acid sequence of which is shown in SEQ ID No. 4. The protein contains L-arginine at position 43. The DNA sequence of the rpsL-1545 allele (SEQ ID No. 3) contains the base guanine at position 128 of the coding region (CDS), which corresponds to position 627 in the sequence shown in SEQ ID No. 3. The DNA sequence of the wild-type gene (SEQ ID No. 1) contains the base adenine at this position

For mutagenesis, conventional mutagenesis processes can be used, using mutagenic substances such as, for example, N-methyl-N'-nitro-N-nitrosoguanidine or ultraviolet light. In vitro methods, such as, for example, a treatment with hydroxylamine (Miller, J. H.: A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1992) or mutagenic oligonucleotides (T. A. Brown: Gentechnologie für Einsteiger [Genetic Engineering for Beginners], Spektrum Akademischer Verlag, Heidelberg, 1993) or the polymerase chain reaction (PCR), such as is described in the handbook by Newton and Graham (PCR, Spektrum Akademischer Verlag, Heidelberg, 1994), can furthermore be used for the mutagenesis.

The rpsL allele according to the invention can also be transferred into suitable strains, inter alia, by the method of gene replacement, such as is described by Schwarzer and Pühler (Bio/Technology 9, 84–87 (1991)) or Peters-Wendisch et al. (Microbiology 144, 915 – 927 (1998)). The corresponding rpsL allele is cloned here in a vector which is not replicative for C. glutamicum, such as, for example, pK18mobsacB or pK19mobsacB (Jäger et al., Journal of Bacteriology 174: 5462-65 (1992)) or pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)) and this is then transferred into the desired host of C. glutamicum by transformation or conjugation. After homologous recombination by means of a first "cross-over" event which effects integration and a suitable second "cross-over" event which effects excision in the target gene or in the target sequence, the incorporation of the mutation is achieved.

In addition, it may be advantageous for the production of L-amino acids to enhance, in particular over-express, one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the citric acid cycle, of the pentose phosphate cycle, of amino acid export and optionally regulatory proteins, in addition to the rpsL gene. The use of

endogenous genes is in general preferred.

"Endogenous genes" or "endogenous nucleotide sequences" are understood as meaning the genes or nucleotide sequences and alleles thereof present in the population of a species.

- 5 Thus, for the preparation of L-lysine, in addition to enhancement of the rpsL gene,

one or more genes chosen from the group consisting of

the dapA gene which codes for dihydridipicolinate synthase (EP-B 0 197 335),

the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),

10 the tpi gene which codes for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),

the pgk gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),

the zwf gene which codes for glucose 6-phosphate dehydrogenase (JP-A-09224661),

15 the pyc gene which codes for pyruvate carboxylase (DE-A-198 31 609),

the mqo gene which codes for malate-quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),

20 the lysC gene which codes for a feed-back resistant aspartate kinase (Kalinowski et al. (1990), Molecular Microbiology 5(5), 1197-204 (1991)),

the lysE gene which codes for the lysine export protein (DE-A-195 48 222),

the zwa1 gene which codes for the Zwa1 protein (DE: 19959328.0, DSM 13115), and

25 the rpoB gene which codes for the β -subunit of RNA polymerase B, shown in SEQ ID No. 5 and 6 can be enhanced, in particular over-expressed.

The term "attenuation" in this context describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or using a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the corresponding gene or enzyme (protein), and optionally combining these measures.

30 By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the starting microorganism.

It may furthermore be advantageous for the production of L-amino acids, in addition to the enhancement of the rpsL gene, for one or more genes chosen from the group consisting of:

5 the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1; DSM 13047),
the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478; DSM 12969),
the poxB gene which codes for pyruvate oxidase (DE: 1995 1975.7; DSM 13114),
the zwa2 gene which codes for the Zwa2 protein (DE: 19959327.2, DSM 13113) to
10 be attenuated, in particular for the expression thereof to be reduced.

In addition to enhancement of the rpsL gene it may furthermore be advantageous for the production of amino acids to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

15 The invention also provides the microorganisms prepared according to the invention, and these can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of production of amino acids. A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).
20 The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

25 Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

5 nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e. g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

10 substances, such as amino acids and vitamins, can be employed in addition to the above-

mentioned substances. Suitable precursors can moreover be added to the culture medium.

The starting substances mentioned can be added to the culture in the form of a single batch.

¹¹ See also the discussion in the previous section.

or can be fed in during the culture in a suitable manner.

15 Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-
20 containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of the desired product has formed. This target is usually reached within 10 hours to 160 hours.

Methods for the determination of L-amino acids are well known to those skilled in the art. The analysis can thus be carried out, for example, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190) by ion exchange chromatography with subsequent ninhydrin derivation, or it can be carried out by reversed phase HPLC, for example as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

A pure culture of the *Corynebacterium glutamicum* strain DM1545 was deposited on 16th January 2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty as DSM 13992.

The process according to the invention is used for the fermentative preparation of amino acids, in particular L-lysine.

EXAMPLES

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

The isolation of plasmid DNA from *Escherichia coli* and all techniques of restriction, Klenow and alkaline phosphatase treatment were carried out by the method of Sambrook et al. (Molecular Cloning. A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA). Methods for transformation of *Escherichia coli* are also described in this handbook.

The composition of the usual nutrient media, such as LB or TY medium, can also be found in Sambrook et al. cited above.

Example 1

Preparation of a Genomic Cosmid Gene Library from *Corynebacterium glutamicum* ATCC 13032

Chromosomal DNA from *Corynebacterium glutamicum* ATCC 13032 is isolated as described by Tauch et al. (1995, Plasmid 33:168-179) and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Code no. 27-0913-02). The DNA fragments are dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, Product Description SuperCos1 Cosmid Vector Kit, Code no. 251301) is cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, Product Description XbaI, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.

The cosmid DNA is then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Code no. 27-0868-04). The cosmid DNA treated in this manner is mixed with the treated ATCC13032 DNA and the

batch is treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture is then packed in phages with the aid of Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217).

5 For infection of the *E. coli* strain NM554 (Raleigh et al. 1988, Nucleic Acid Research 16:1563-1575) the cells are taken up in 10 mM MgSO₄ and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library are carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190) with 100 10 mg/l ampicillin. After incubation overnight at 37°C, recombinant individual clones are selected.

Example 2

Isolation and Sequencing of the *rpsL* Gene

15 The cosmid DNA of an individual colony is isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-02). The DNA 20 fragments are dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range of 1500 to 2000 bp are isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, 25 Germany).

The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, Holland, Product Description Zero Background Cloning Kit, Product No. K2500-01), is 25 cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 is carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture being 30 incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture is then electroporated (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) into the *E. coli* strain DH5 α MCR (Grant, 1990, Proceedings of the National Academy of Sciences

U.S.A., 87:4645-4649) and plated out on LB agar (Lennox, 1955, *Virology*, 1:190) with 50 mg/l zeocin.

The plasmid preparation of the recombinant clones is carried out with a Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing is carried out by the 5 dideoxy chain termination method of Sanger et al. (1977, *Proceedings of the National Academy of Sciences U.S.A.*, 74:5463-5467) with modifications according to Zimmermann et al. (1990, *Nucleic Acids Research*, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) is used. The separation by gel electrophoresis and analysis of the sequencing reaction are 10 carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained are then processed using the Staden program package 15 (1986, *Nucleic Acids Research*, 14:217-231) version 97-0. The individual sequences of the pZero1 derivatives are assembled to a continuous contig. The computer-assisted coding region analysis is prepared with the XNIP program (Staden, 1986, *Nucleic Acids Research* 14:217-231).

The resulting nucleotide sequence is shown in SEQ ID No. 1. Analysis of the 20 nucleotide sequence shows an open reading frame of 383 base pairs, which is called the rpsL gene. The rpsL gene codes for a protein of 127 amino acids.

The DNA section lying upstream of SEQ ID No. 1 was identified in the same way, this section being shown in SEQ ID No. 7. The rpsL gene region extended by SEQ ID No. 7 is shown in SEQ ID No. 8.

Example 3

25 Amplification and Sequencing of the DNA of the rpsL Allele of Strain DM1545

The *Corynebacterium glutamicum* strain DM1545 was prepared by multiple, non-directed mutagenesis, selection and mutant selection from *C. glutamicum* ATCC13032. The strain is methionine-sensitive.

30 From the strain DM1545, chromosomal DNA is isolated by conventional methods (Eikmanns et al., *Microbiology* 140: 1817 - 1828 (1994)). With the aid of the polymerase

chain reaction, a DNA section which carries the rpsL gene or allele is amplified. On the basis of the sequence of the rpsL gene known for C. glutamicum from example 2, the following primer oligonucleotides are chosen for the PCR:

rpsL-1 (SEQ ID No. 10):

5' cag ctc tac aag agt gtc ta 3'

rpsL-2 (SEQ ID No. 11):

5' tgg tcg tgg tct tac cag ca 3'

The primers shown are synthesized by MWG Biotech (Ebersberg, Germany) and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA section of approx. 1.78 kb in length, which carries the rpsL allele.

The amplified DNA fragment of approx. 1.78 kb in length which carries the rpsL allele of the strain DM1545 is identified by electrophoresis in a 0.8% agarose gel, isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

The nucleotide sequence of the amplified DNA fragment or PCR product is determined by sequencing by MWG Biotech (Ebersberg, Germany). The sequence of the PCR product is shown in SEQ ID No. 3. The amino acid sequence of the associated ribosomal protein S12 resulting with the aid of the Patentin program is shown in SEQ ID No.

20 4.

At position 128 of the nucleotide sequence of the coding region of the rpsL allele of strain DM1545, that is to say at position 627 of the nucleotide sequence shown in SEQ ID No. 3, is the base guanine. At the corresponding position of the wild-type gene is the base adenine (SEQ ID No. 1).

25 At position 43 of the amino acid sequence of the ribosomal protein S12 of strain DM1545 is the amino acid arginine (SEQ ID No. 4). At the corresponding position of the wild-type protein is the amino acid lysine (SEQ ID No. 2).

Example 4

30 Replacement of the rpsL Wild-type Gene Of
Strain DSM5715 by the rpsL-1545 Allele

4.1 Isolation of a DNA fragment which carries the rpsL-1545 allele

From the strain DM1545, chromosomal DNA is isolated by the conventional methods (Eikmanns et al., Microbiology 140: 1817 - 1828 (1994)). A DNA section which carries the rpsL-1545 allele which contains the base guanine at position 128 of the coding region (CDS) instead of the bases adenine contained at this position in the wild-type gene is amplified with the aid of the polymerase chain reaction. On the basis of the sequence of the rpsL gene known for *C. glutamicum* from example 2, the following primer oligonucleotides are chosen for the polymerase chain reaction :

5 rpsL_XL-A1 (SEQ ID No. 12):

5' ga tct aga-ggt tgc cgg taa tcc tgt tg 3'

10 rpsL_XL-E1 (SEQ ID No. 13):

5' ga tct aga-cgc agg ctg cca gct tat tc 3'

15 The primers shown are synthesized by MWG Biotech (Ebersberg, Germany) and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA section approx. 1.59 kb in length which carries the rpsL-1545 allele (SEQ ID No. 9). The primers moreover contain the sequence for a cleavage site of the restriction endonuclease XbaI, which is marked by underlining in the nucleotide sequence shown above. The amplified DNA fragment of approx. 1.59 kb in length which carries the rpsL-1545 allele is cleaved with the restriction endonuclease XbaI, identified by electrophoresis in a 0.8% agarose gel and then isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

4.2 Construction of the exchange vector pK18mobsacB_rpsL-1545

20 The approximately 1.58 kb long DNA fragment cleaved with the restriction endonuclease XbaI, which contains the rpsL-1545 allele and is described in example 4.1, is incorporated by means of replacement mutagenesis with the aid of the sacB system described by Schäfer et al. (Gene, 14, 69-73 (1994)) into chromosome of the *C. glutamicum* strain DSM5715. This system enables preparation and selection of allele exchanges which take place by homologous recombination.

25 The mobilizable cloning vector pK18mobsacB is digested with the restriction enzyme XbaI and the ends are dephosphorylated with alkaline phosphatase (Alkaline Phosphatase,

Boehringer Mannheim, Germany). The vector prepared in this way is mixed with the rpsL-1545 fragment approx. 1.58 kb in size and the mixture is treated with T4 DNA ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain S17-1 (Simon et al., Bio/Technologie 1: 784-791, 1993) is then transformed with the ligation batch (Hanahan, In. DNA cloning. A Practical Approach. Vol. 1, IRL-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 25 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage with the enzyme PstI and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacB_rpsL-1545 and is shown in figure 1.

4.3 Integration of the vector pK18mobsacB_rpsL-1545 in DSM5715 and allele exchange

The vector pK18mobsacB_rpsL-1545 mentioned in example 4.2 is transferred by conjugation by the protocol of Schäfer et al. (Journal of Microbiology 172: 1663-1666 (1990)) into C. glutamicum strain DSM5715. The vector cannot replicate independently in DSM5715 and is retained in the cell only if it is present integrated in the chromosome as the consequence of a recombination event. Selection of transconjugants, i.e. clones with integrated pK18mobsacB_rpsL-1545, is made by plating out the conjugation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which is supplemented with 15 mg/l kanamycin and 50 mg/l nalidixic acid. Kanamycin-resistant transconjugants are plated out on LB agar plates with 25 mg/l kanamycin and incubated for 24 hours at 33 °C. A kanamycin-resistant transconjugant is called DSM5715::pK18mobsacB_rpsL-1545. By integration of the vector, in addition to the rpsL wild-type gene it carries the rpsL-1545 allele in the chromosome. For selection of mutants in which excision of the plasmid has taken place as a consequence of a second recombination event, cells of the strain DSM5715::pK18mobsacB_rpsL-1545 are cultured for 30 hours unselectively in LB liquid medium and then plated out on LB agar with 10% sucrose and incubated for 16 hours.

The plasmid pK18mobsacB_rpsL-1545, like the starting plasmid pK18mobsacB, contains, in addition to the kanamycin resistance gene, a copy of the sacB gene which codes for levan sucrase from *Bacillus subtilis*. The expression which can be induced by sucrose leads to the formation of levan sucrase, which catalyses the synthesis of the product levan, which is toxic to *C. glutamicum*. Only those clones in which the integrated pK18mobsacB_rpsL-1545 has excised as the consequence of a second recombination event therefore grow on LB agar. Depending on the position of the second recombination event with respect to the mutation site, allele exchange or incorporation of the mutation takes place with the excision, or the original copy remains in the chromosome of the host.

Approximately 40 to 50 colonies are tested for the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin". In 4 colonies which show the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin", a region of the rpsL gene spanning the rpsL-1545 mutation is sequenced, starting from the sequencing primer rL_1 (SEQ ID No. 14), by GATC Biotech AG (Constance, Germany) to demonstrate that the mutation of the rpsL-1545 allele is present in the chromosome. The primer rL-1 used is synthesized for this by GATC:

rL_1 (SEQ ID No. 14):

5' atg agg ttg tcc gtg aca tg 3'

A clone which contains the base guanine at position 128 of the coding region (CDS) of the rpsL gene and thus has the rpsL-1545 allele was identified in this manner. This clone was denoted strain DSM5715_rpsL-1545.

Example 5

Preparation of Lysine

The *C. glutamicum* strains DSM5715::pK18mobsacB_rpsL-1545 and DSM5715rpsL-1545 obtained in example 4 are cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant is determined.

For this, the strains are first incubated on an agar plate for 24 hours at 33°C. Starting from this agar plate culture, in each case a preculture is seeded (10 ml medium in a 100 ml conical flask). The medium MM is used as the medium for the precultures. The precultures are incubated for 24 hours at 33°C at 240 rpm on a shaking machine. In each case a main culture

is seeded from these precultures such that the initial OD (660 nm) of the main cultures is 0.1. The Medium MM is also used for the main cultures.

Medium MM

5	CSL	5 g/l
	MOPS	20 g/l
	Glucose (autoclaved separately)	50 g/l
	Salts:	
10	$(\text{NH}_4)_2\text{SO}_4$	25 g/l
	KH_2PO_4	0.1 g/l
10	$\text{MgSO}_4 * 7 \text{H}_2\text{O}$	1.0 g/l
	$\text{CaCl}_2 * 2 \text{H}_2\text{O}$	10 mg/l
	$\text{FeSO}_4 * 7 \text{H}_2\text{O}$	10 mg/l
15	$\text{MnSO}_4 * \text{H}_2\text{O}$	5.0mg/l
	Biotin (sterile-filtered)	0.3 mg/l
15	Thiamine * HCl (sterile-filtered)	0.2 mg/l
	L-Leucine (sterile-filtered)	0.1 g/l
20	CaCO_3	25 g/l

The CSL (corn steep liquor), MOPS (morpholinopropanesulfonic acid) and the salt solution are brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions, as well as the CaCO_3 autoclaved in the dry state, are then added.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Culturing is carried out at 33°C and 80% atmospheric humidity.

After 72 hours, the OD is determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed is determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivation with ninhydrin detection.

The result of the experiment is shown in table 1.

Table 1

Strain	OD (660 nm)	Lysine HCl
DSM5715	8.2	13.57
DSM5715::pK18mobsacB _rpsL-1545	9.2	15.28
DSM5715rpsL-1545	7.9	14.74

5

10

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

All publications cited above are incorporated herein by reference.

This application is based on German patent application serial Nos. 101 07 230.9, filed on February 16, 2001; and German patent application serial No. 101 62 386.0, filed December 19, 2001, both of which are incorporated herein by reference in their entirety.